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(71) Applicant: MEDICAL RESEARCH COUNCIL
20 Park Crescent
London W1 4AL(GB)

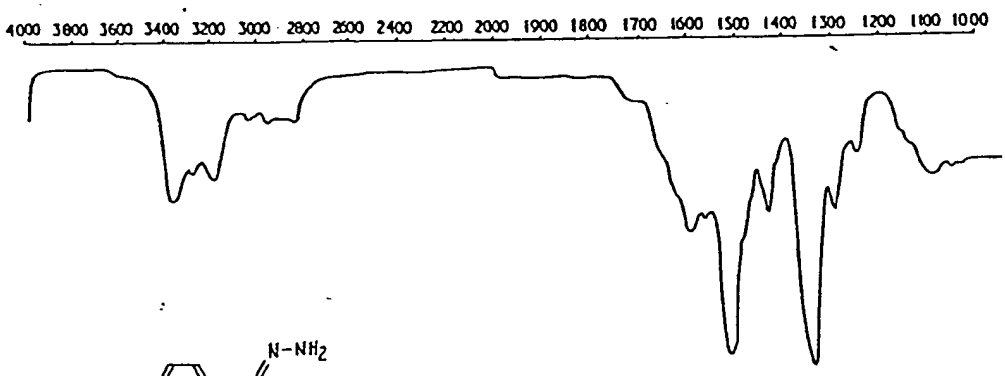
(72) Inventor: Trentham, David Rostron
c/o Medical Research Council 20 Park Crescent
London W1N 4AL(GB)

(72) Inventor: Reid, Gordon Paterson
c/o Medical Research Council 20 Park Crescent
London W1N 4AL(GB)

(74) Representative: Pennant, Pyers et al,
Stevens, Hewlett & Perkins 5 Quality Court Chancery Lane
London, WC2A 1HZ(GB)

(54) Photo-labile protecting agents and method.

(57) The invention provides diazo compounds having the formula XYCNN and their parent hydrazones, where X is an ortho-nitro aromatic group such as o-nitrophenyl and Y is a hydrocarbon group such as methyl. The hydrazones may be prepared by reacting the corresponding ketone with hydrazine and the diazo compounds by hydrazone oxidation. The diazo compound may be reacted with an organic compound having a reactive hydrogen atom such as phosphate, thiophosphate, phosphonate, carboxyl and phenol, to form a caged compound from which the original organic compound can be released by photolysis, eg in situ in a biological system. Preferred organic compounds are ATP and other nucleotides.



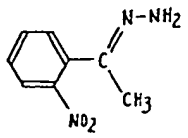


FIG. 1

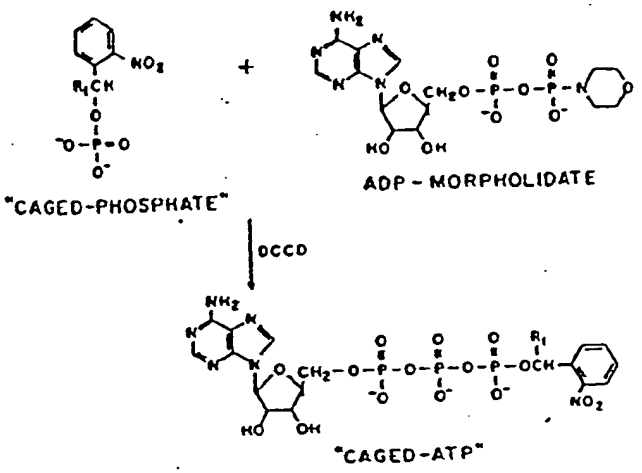
EP 0 233 403 A1

PHOTO-LABILE PROTECTING AGENTS AND METHOD

This invention relates to certain diazo compounds, many of which are new compounds per se, and to their use as photo-labile protecting agents for organic compounds. The invention will provide a useful new way for preparing protected or 'caged' organic compounds which can be introduced into biological systems and there released by means of light radiation. But the invention is likely to be applicable also in other areas, for example in organic synthesis and purification.

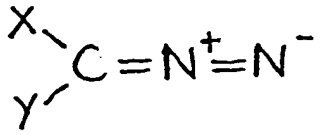
2-nitrobenzyl derivatives have been used for many years as photo-labile protecting groups in synthetic organic chemistry. Indeed, 2-nitrobenzaldehyde tosylhydrazone, a precursor of 2-nitrophenyl-diazomethane, is commercially available for this purpose. Such compounds are not suitable for use in biological systems, because photolysis gives rise to a reactive benzaldehyde compound and in certain cases the compounds themselves are susceptible to attack by endogenous thiols.

A caged derivative of adenosine 5'-triphosphate (ATP) has been described, in which the caging group is 1-(2-nitro)phenylethyl, and has been shown to act as photo-labile source of ATP (Kaplan et al (1978) Biochemistry 17, pages 1929 to 1935). Preparation of caged ATP involves reaction of ADP morpholidate with 1-(2-nitro)phenylethyl phosphate by the following reaction.



The method is labour-intensive and gives rise to low yields. In particular, it is difficult to make radioactively labelled caged ATP, such as is constantly required for biological experiments, although this has been achieved (Ferenczi et al, J. Physiol.(1984), 352, pages 575 to 599). There has long been a need for a better method of making caged ATP, and in particular for a method that can readily be used to make caged radiolabelled ATP.

In one aspect this invention provides a diazo compound having the formula

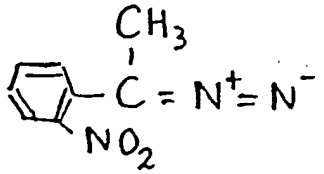


where X is an optionally substituted aromatic group which carries a $-NO_2$ group in the ortho-position, and Y is an optionally substituted hydrocarbon group. As described below, these compounds can be reacted with ATP, or with another organic compound having a reactive hydrogen atom, to provide a caged compound.

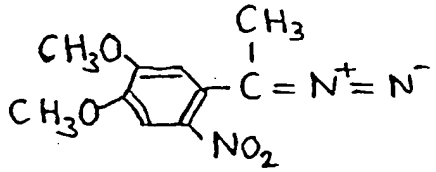
The group X may be 2-nitrophenyl. The phenyl group may carry hydrocarbon or other electron-donating

substituents to assist rapid photolysis. Or the phenyl group may carry one or more groups such as alkoxy groups to increase absorption of light during photolysis or to shift the optimum wavelength for photolysis to longer wavelengths. Examples of diazo compounds falling within the above definition are:-

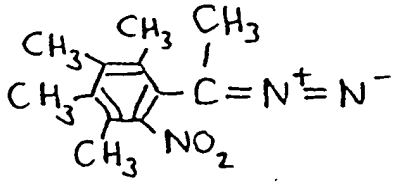
1-(2-nitro)phenyl-diazoethane (I)

I 

1-(2-nitro-4,5-dimethoxy)phenyl-diazoethane (II)

II 

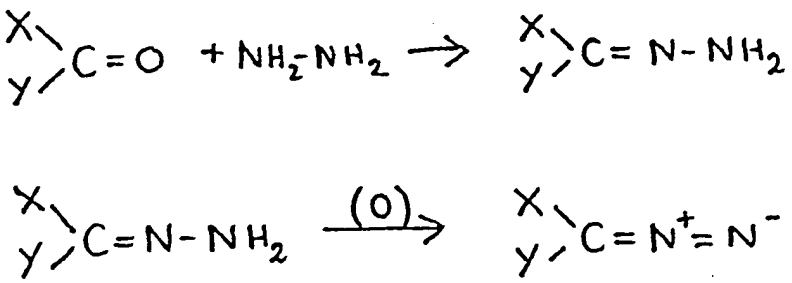
1-(2-nitro-3,4,5,6-tetramethyl)phenyl-diazoethane (III)

III 

To the best of our knowledge these three, together with others falling within the above definition, are new compounds. Generally they lead to caged compounds with an asymmetric carbon atom. If the parent compound is a chiral molecule (such as ATP, see above), this will lead to a caged compound (e.g. caged ATP) that exists as two diastereoisomers. (In the case of caged ATP these two diastereoisomers have been separated and shown to have identical

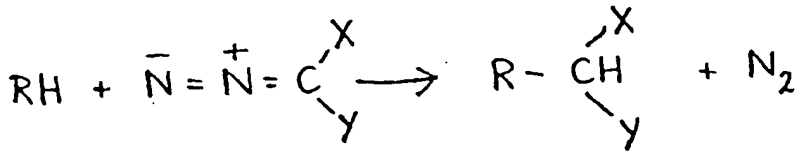
photochemical properties, so that, in general, separation of diastereoisomers is not necessary).

The diazo compounds may be prepared from the corresponding ketones by the following two-step method:-



In the first step, the ketone is reacted with hydrazine hydrate to form the corresponding hydrazone. These hydrazone intermediates are believed to be new compounds, and form a further aspect of this invention. Since they are more stable than the corresponding diazo compounds, to which they may readily be converted by oxidation, they provide a convenient form in which the diazo compounds may be stored, transported and sold. In the second step, the hydrazone may be oxidized to the corresponding diazo compound using known chemistry.

According to another aspect, the invention provides a method which comprises reacting an organic compound having a reactive hydrogen atom (or an organic compound in its conjugate base form) with a diazo compound as defined above. The reaction may be shown as follows, where RH is the organic compound concerned:



The reactive hydrogen atom may for example be part of a group selected from phosphate, thiophosphate, phosphonate, carboxyl and phenol. The organic compound may be used in its conjugate base form, that is to say with the reactive hydrogen atom ionized, though the protonated form is generally believed to be the reactive species. The reaction proceeds easily under a wide range of conditions. For hydrophilic compounds of biological interest, the reaction is preferably performed with shaking in a water/organic solvent mixture, using for example chloroform or ether as the water-immiscible organic solvent. For example, reaction with ATP and other nucleotides in a shaken water/chloroform mixture at ambient temperature overnight is found to give the caged compounds in high yield and with excellent selectivity. Reaction temperature is not critical. For organic compounds in which the reactant or product is not soluble or dispersible in water, it may be preferable to use a single-phase organic reaction medium. Examples 13 and 14 illustrate this. On occasion (for example in the case of cyclic nucleotides listed below) a single-phase organic reaction is desirable because water competes with the reactant for the diazo compound. The caged product is readily recovered from the reaction mixture and purified, for example by means of HPLC. On occasion more than one group is caged. If it is desired that only one group should be caged, standard chemical procedures can be used to obtain the desired product. Examples 12 and 13 illustrate this. Caged compounds prepared by these methods may, if necessary, be further modified. Example 15 illustrates this. The following is a list of biologically significant compounds that can be caged by the method of this invention.

1. Phosphates, thiophosphates and phosphonates

ribonucleotides
e.g. adenosine 5' triphosphate
guanosine 5' diphosphate
cytosine 5'monophosphate

deoxyribonucleotides
e.g. deoxyadenosine 5'triphosphate
deoxythymidine 5' diphosphate
deoxyguanosine monophosphate

nucleotide analogues
e.g. 2',3'-dideoxythymidine 5'-triphosphate
cytosine-β-D-arabinofuranoside 5'-triphosphate
1,N^6-ethenoadenosine 5'-triphosphate
1,N^6-etheno-2-aza-adenosine 5'-triphosphate
adenylyl-imidodiphosphate (ATP (β,γ-NH))
adenosine 5'-O-(3-thiotriphosphate) (ATP(γS))
adenylyl-(β,γ-methylene) diphosphonate (ATP(β,γ-CH_2))
GTP(β,γ-NH)
GTP(γS)
GTP(β,γ-CH_2)

oligonucleotides

cyclic nucleotides
e.g. cyclic adenosine 3',5' monophosphate (cAMP),
cGMP,
cCMP

cyclic nucleotide analogues
e.g. cyclic adenosine 3',5'-monophosphothioate,
cyclic guanosine 3',5'-monophosphothioate
cyclic formycin 3',5' monophosphate

Sugar phosphates
e.g. glucose 1-phosphate,
glucose 6-phosphate

inositol trisphosphate
and related compounds
e.g. phosphatidylinositolbis-phosphate (PIP_2),
phosphatidylinositol monophosphate (PIP_1),
phosphatidylinositol,
inositol tetraphosphate (IP_4),
inositol bisphosphate (IP_2),
inositol phosphate (IP_1),
inositol 1,4,5-trisphosphate,
inositol 1,3,4-trisphosphate

Phosphates in proteins
and peptides

Phosphonate receptor agonists and antagonists
e.g. 2-amino-4-phosphono-butyric acid,
2-amino-4-phosphono-valeric acid

vitamin derivatives
e.g. pyridoxal phosphate

Phosphoadenosylphosphosulphate (PAPS)

2. Carboxyl and phenolic groups,

amino acids
e.g. glycine

peptides
e.g. enkephalins

neurotransmitters
e.g. γ-aminobutyric acid

catecholamines

fatty acids

thromboxanes

prostaglandins

fluorescein

Of course, where a radioactively labelled caged compound is required, a radioactively labelled starting compound with a reactive hydrogen atom would be used. The synthetic method described here is far easier and has much higher yield for radiochemicals than the earlier method used for caged ATP (Ferenczi et al., J. Physiol. 352, 575-599, 1984).

The resulting caged compounds are in many instances known,as are the conditions required for their photolysis. Thus for example, caged ATP and its photolysis are described in the two references quoted above. Light from 300 to 350 nm is suitable for this photolysis, such as may be generated by a xenon-arc flash lamp or a 347 nm frequency doubled ruby laser. A 10 millisecond exposure from a filtered (300 to 350 nm) mercury or xenon-arc lamp source is perfectly adequate when high time resolution is not required. The quantum yield for photolysis of caged ATP is about 0.7, and for any caged phosphate diester is in the range 0.3 to 0.7. When diazo compounds having alkoxy, e.g. methoxy, groups have been used, an energy input of from 1 to 5 mJ is typically sufficient for photolysis; when the diazo compound did not contain methoxy groups, an energy

input of from 10 to 50 mJ may typically be required.

Applications of the method of this invention all involve the following steps:-

a) Providing a caged organic compound which is the reaction product of an organic compound having a reactive hydrogen atom with a diazo compound as defined.

b) Doing something to the caged organic compound, for example changing its chemical identity or its environment.

c) Illuminating the caged compound to effect photolysis.

Several applications of this general technique may be noted:-

i) The caged compound is introduced to a biological system, and is there photochemically decomposed so as to form the organic compound in situ in the biological system. Techniques of this kind are well known and will not be described here. The contribution of this invention is to provide a better method, and in many cases perhaps the only feasible method, of making caged compounds.

ii) The technique may be used to purify a desired compound. For example, a biological mixture may be reacted with one of the diazo compounds, so as to form a caged compound which may then be separated, e.g. by HPLC, from the remainder of the mixture. After recovery from the column and desalting the solution, the pure compound may simply be recovered by photolysis.

iii) The technique may be used in organic synthesis. For example, a reactive group of a starting organic compound may be caged or protected by reaction with a diazo compound as defined. Then the organic compound may be modified by reaction of some other part of the molecule so as to form a protected product

compound,which is subsequently recovered by photolysis. This technique is well known in the art and other photolysable protecting groups have been proposed for the purpose. The contribution of this invention is to provide a new photolysable protecting compound and method which may have advantages in certain instances.

Reference is directed to the accompanying drawings, in which:-

Figure 1. is an infrared spectrum of the product of Example 1, o-nitroacetophenone hydrazone.

Figure 2. is an I.R. spectrum of the product of Example 2, 1-(2-nitrophenyl)-diazoethane.

Figure 3. is an HPLC analysis using C-18 (solvent 10 mM inorganic phosphate at pH 5.5, 85%: methanol 15%) of the product of Example 3; absorbent detection at 254 mm; and

Figure 4. is HPLC analysis of the same product after photolysis.

The following examples illustrate the invention. In all cases the amounts of reactants used can be readily scaled up or down.

Example 1

Synthesis of o-nitroacetophenone hydrazone

o-nitroacetophenone hydrazone was prepared by an acid catalysed reaction of o-nitroacetophenone with hydrazine hydrate.

(0.112 moles) 5.62g hydrazine hydrate and (0.056 moles) 3.2 ml glacial acetic acid were added to (0.05 moles) 8.26g 0-nitroacetophenone in 100 ml ethanol and heated under reflux for 2.5 hour. The ethanol was evaporated off under vacuum and the product examined by I.R.

(Figure 1). Disappearance of a 1690 cm^{-1} band in the I.R. due to C=O stretch was taken as evidence of complete reaction.

The solution was evaporated to dryness and dissolved in diethyl ether. A precipitate of, probably, hydrazine acetate was filtered off and the ether solution containing the hydrazone used in next reaction.

Example 2

Synthesis of1-(2-nitrophenyl)diazoethane

The o-nitroacetophenone hyrazone was oxidised with activated manganese dioxide by the method of Jugelt and Berseck. The ether solution was stirred with 11.3 g manganese dioxide for 3 hours at room temperature in the dark. The manganese dioxide was filtered off and the ether solution partitioned with water adjusted to pH 7 with sodium bicarbonate. The separated ether phase was dried with magnesium sulfate and stored in a tightly stoppered flask covered with tin foil to shield from the light.

The wine coloured ether solution was examined after evaporating the ether by I.R. (Figure 2). A peak at 2050cm^{-1} was observed and attributed to the diazo group. The product dissolved in $CHCl_3$ was used directly in the subsequent esterifications.

Jugelt W and Berseck TETRAHEDRON 26, 5581 (1970)

Example 3

Synthesis of 1-(2-nitrophenyl)ethyl esters

ATP was treated with Dowex H^+ to yield the H^+

form which was adjusted to pH 4-5 with NaOH. 3ml of an aqueous solution containing from 1-100 u mole of the phosphate ester was stirred vigorously with 3ml of a $CHCl_3$ solution containing 0.4 m mole 1-(2-nitrophenyl)diazoethane at room temperature for 15 hour. The product was analyzed by HPLC (generally C-18 reverse phase column) and shown to be esterified in 7' 100% yield. After the $CHCl_3$ phase had been separated off, the phosphate diester was purified by C-18 reverse phase chromatography typically in 10 mM inorganic phosphate pH 5.5, Methanol (85:15 v/v). The solution containing the pure compound is adjusted to pH 7.5 and is direc y loaded onto a DEAE column and eluted using a gradien of triethylamine bicarbonate in water. The solution containing the pure compound is rotary evaporated tc remove excess triethylamine/bicarbonate leaving the pure compound. Care is taken to shield the compounc from daylight, though in practice insignificant photolysis occurs in normal daylight. Figure 3 shows the result of HPLC analysis of the initial product before purification. Figure 4 shows HPLC analysis c the mixture produced on photolysis of the product. Th caged ATP appears as a double peak (due to stereo isomerism) at about 6 minutes; the ATP formed on photolysis appears as a peak around 2 minutes.

Other caged compounds were prepared and characterized by the general method described in Example 3.

Examples

4 P-1-(2-nitro)phenylethylguanosine 5'-phosphate (caged 5'-GMP)

5 P^3-1-(2-nitro)phenylethyl-2',3'-dideoxythymidine 5'-triphosphate (caged dideoxy TT

6 P^3-1-(2-nitro)phenylethylcytidine 5'-triphosphate (caged CTP)

7 P^3-1-(2-nitro)phenylethyladenosine 5'-O-(3-thiotriphosphate) (caged ATP(γS)) (In caged ATP(γS) the caged group is attached to the ATP(γS) either through sulphur or non-bridging oxygen atom bonded to γ-phosphorus atom of ATP. The'S-caged ATP(γS)' is separable from the 'O-caged ATP(γS)' by DEAE-anion exchange chromatography. The compounds have similar photochemical properties, each forming ATP(γS) on photolysis.)

8 P^3-1-(2-nitro)phenylethylguanosine 5'-O-(3-thiotriphosphate) (caged GTP(γS))

9 P^3-1-(2-nitro)phenylethyladenylyl-imidodiphosphate (caged ATP(β,γ-NH))

10 P^3-1-(2-nitro)phenylethylguanylyl-imidodiphosphate (caged GTP(β,γ-NH))

11 P^3-1-(2-nitro)phenylethyl-[2-^{3}H]adenosine 5'-triphosphate ([2-^{3}H]caged ATP)

Example 12

Inositol, 1,4,5-trisphosphate was reacted with 1-(2-nitrophenyl)diazoethane by the method of Example 3. There was obtained a mixture of caged inositol trisphosphates, including the 1- and 4- and 5- single caged compounds, the 1,4- and the 1,5- and the 4,5- doubly caged compounds, and the 1,4,5-triply caged compound. Reaction for only 4 hours favoured formation of the mixed singly caged compounds. Reaction for 15 hours favoured formation of the triply caged compounds.

In the reaction to form the single caged compound the 1-compound forms about four times more rapidly than the 4- or 5-compound. On the other hand if the 1,4,5-triply caged compound is partially photolyzed the

1-, 4- and 5-compounds are each formed in equal amounts. Thus to form the 1-compound the straightforward procedure is used, while to form the 4- and 5- compounds it is preferably to photolyze partially the 1,4,5-triply caged compound. The single caged isomers may be separated by anion exchange chromatography.

Example 13

Fluorescein was reacted in dimethylsulphoxide with 1-(2-nitro-4,5-dimethoxyphenyl)diazoethane as in Example 3 except for the change of solvent. The product was caged on both its carboxy and phenoxy group. The caged group was removed from the carboxy group by leaving the material in 0.1N sodium hydroxide in dimethyl sulphoxide water at 55°C for 30 minutes, leaving fluorescein caged on just the phenoxy group. The compound was purified by preparative thin layer chromatography.

Example 14

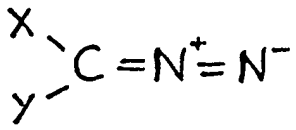
P-1-(2-nitro)phenylethyl cyclic adenosine 3',5'monophosphate: Cyclic adenosine 3'5'-monophosphate was reacted in dimethyl sulphoxide with 1-(2-nitrophenyl)diazoethane as in Example 3 except for the change of solvent. The caged product was purified by preparative thin layer chromatography.

Example 15

P^3-1-(2-nitro)phenylethyl-1-N^6-ethenoadenosine 5'-triphosphate: Caged ATP (Example 3) was treated with chloroacetaldehyde as described by Secrist, Barrio, Leonard and Weber (Biochemistry 11, 3499-3506, 1972) to form the desired product which was then isolated according to procedures outlined in Example 3.

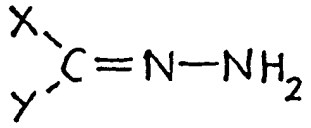
CLAIMS

1. A diazo compound having the formula



where X is an optionally substituted aromatic group which carries a NO_2 group in the ortho-position and Y is an optionally substituted hydrocarbon group.

2. A hydrazone compound having the formula



where X and Y are as defined in claim 1.

3. A compound as claimed in claim 1 or claim 2, wherein X is o-nitrophenyl.

4. A compound as claimed in any one of claims 1 to 3, wherein Y is methyl.

5. A method which comprises reacting an organic compound having a reactive hydrogen atom, or an organic compound in the conjugate base form, with a diazo compound as claimed in any of claims 1, 3 or 4.

6. A method as claimed in claim 5, wherein the reactive hydrogen atom is part of a group selected from phosphate, thiophosphate, phosphonate, carboxyl, and phenol.

7. A method as claimed in claim 5 or claim 6, wherein the reaction is performed in a shaken mixed water/organic solvent system.

8. A method which comprises photochemically decomposing the reaction product of any one of claims 5

to 7.

9. A method comprising the steps:-

providing a caged organic compound which is the reaction product of an organic compound having a reactive hydrogen atom with a diazo compound as claimed in any one of claims 1, 3 or 4,

introducing the caged organic compound into a biological system and,

photochemically decomposing the caged organic compound so as to form the organic compound in situ in the biological system.

10. A purification method comprising the steps:-

providing a starting mixture containing an organic compound having a reactive hydrogen atom and reacting the said organic compound with a diazo compound as claimed in any one of claims 1, 3 or 4, so as to form a protected compound in the mixture,

subjecting the mixture to chromatography and recovering the protected organic compound in purified form, and

photochemically decomposing the protected compound and recovering the purified organic compound.

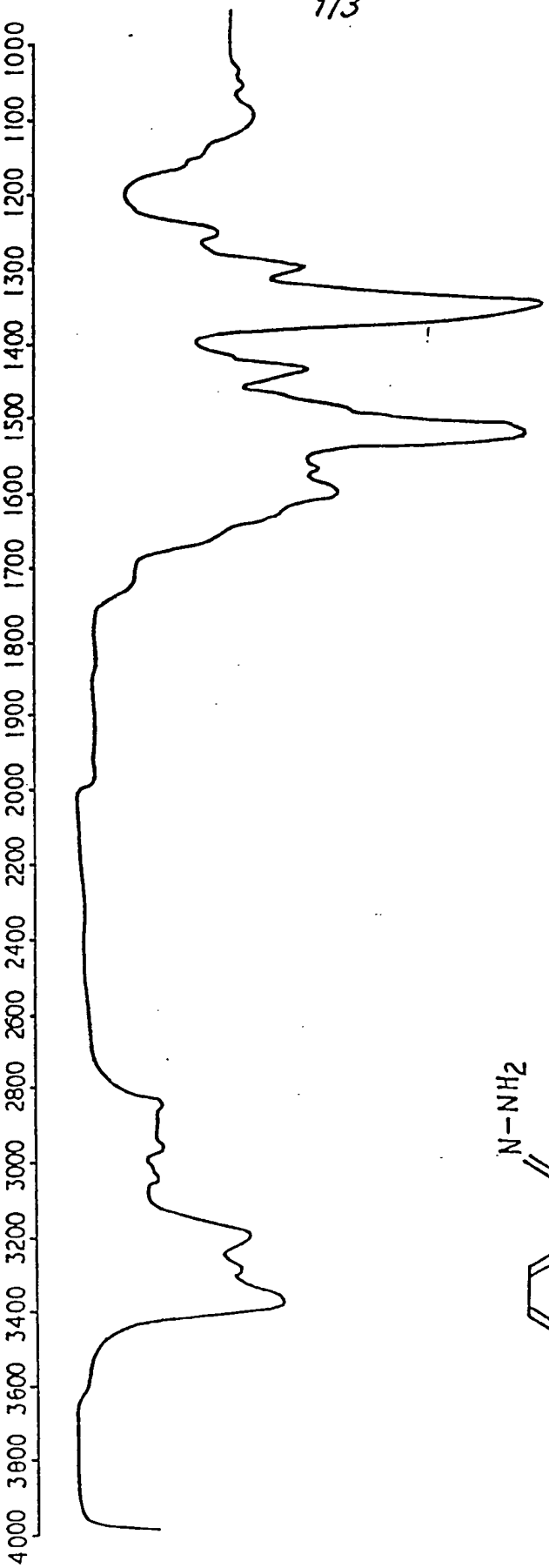
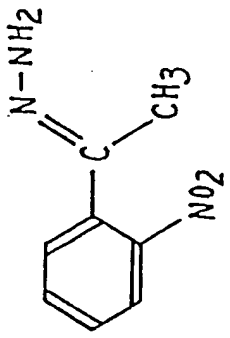


FIG. 1



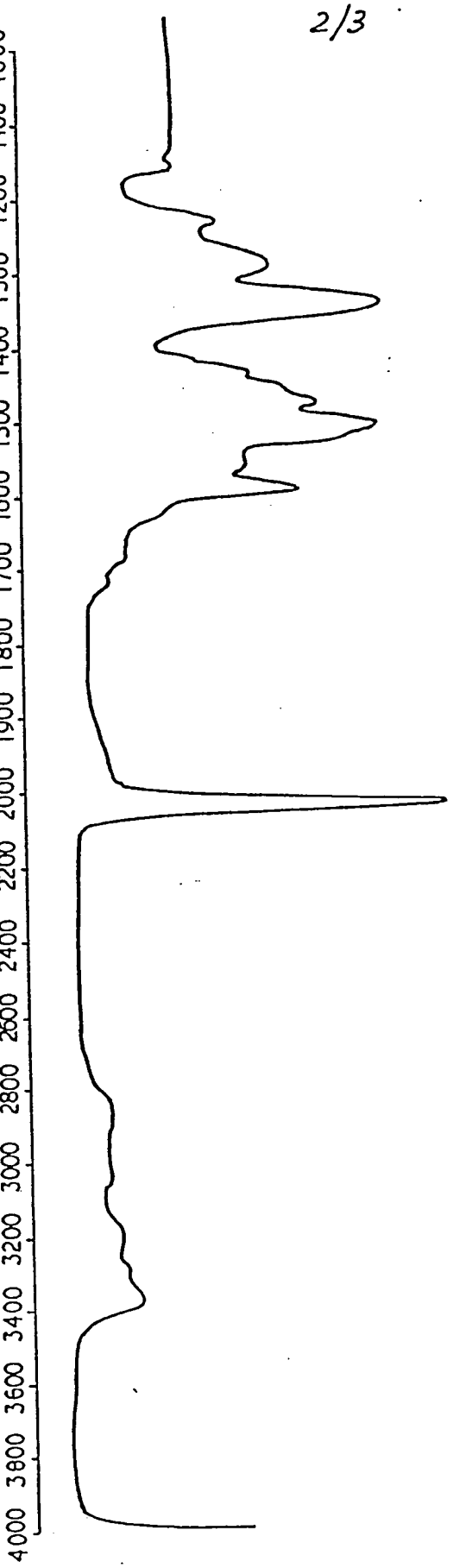
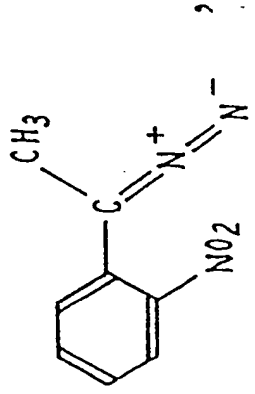

4000 3800 3600 3400 3200 3000 2800 2600 2400 2200 2000 1900 1800 1700 1600 1500 1400 1300 1200 1100 1000

FIG.2


CH3
C
N+
N−
NO2

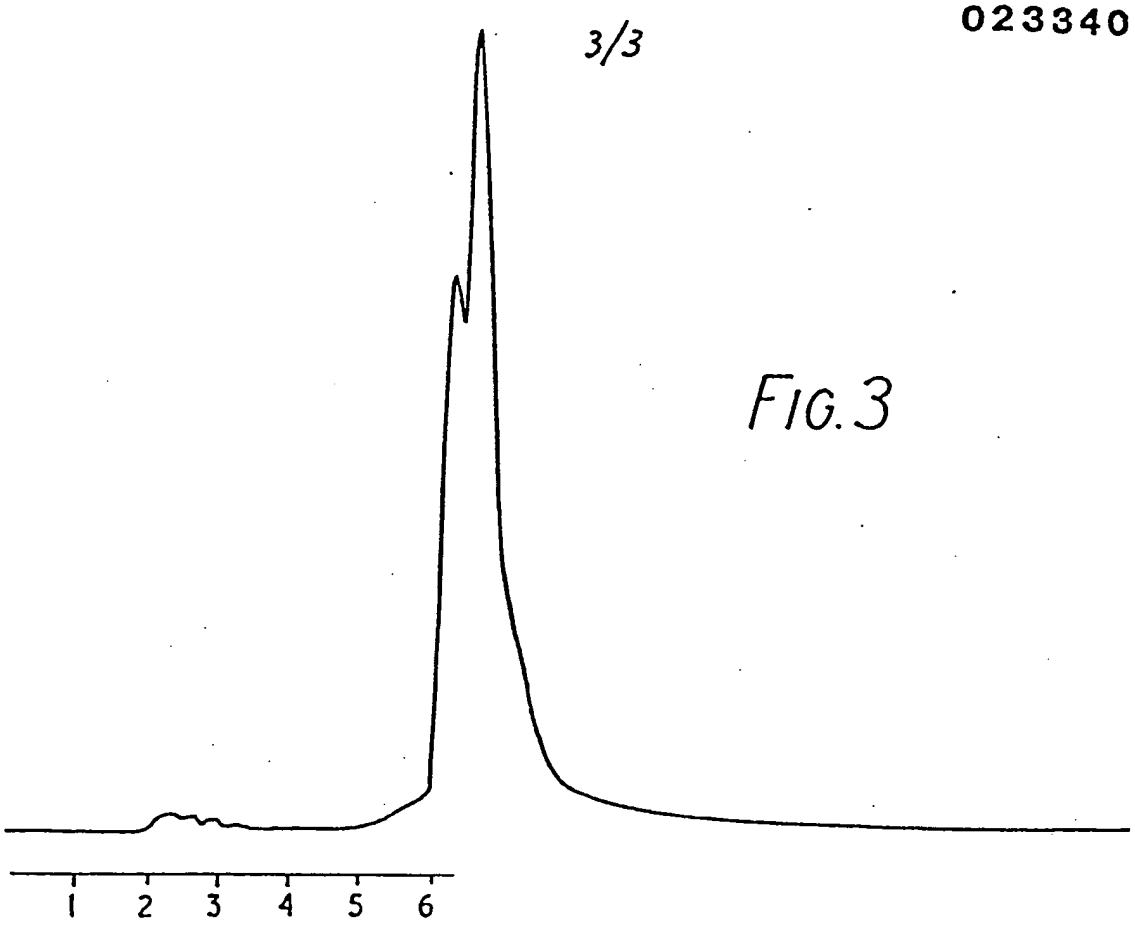


FIG. 3

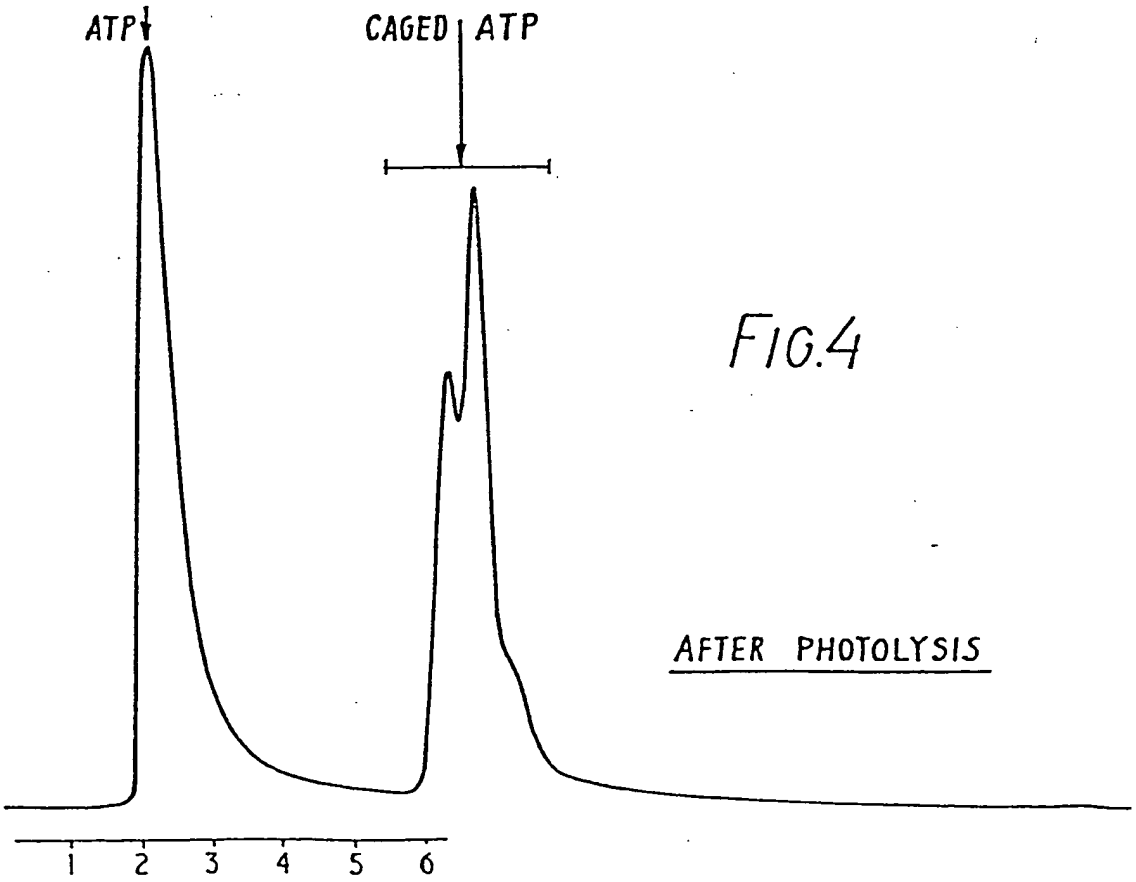


FIG. 4

AFTER PHOTOLYSIS

0233403

Application number

European Patent Office

EUROPEAN SEARCH REPORT

EP 86309271.4

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl 4)
P,A	EP - A1 - 0 187 039 (MORGAN, LEE ROY) * Claims 1,12,18 * --	1,2,5	C 07 C 113/00 C 07 C 109/16 C 07 H 19/20 C 07 B 63/02 B 01 J 19/12
A	PATENT ABSTRACTS OF JAPAN, unexamined applications, C section, vol. 3, no. 147, December 5, 1979 THE PATENT OFFICE JAPANESE GOVERNMENT page 98 C 66 * Kokai no. 54-125 620 (SAGAMI CHUO KAGAKU KENKY USHO) * --	1,2,5	
A	DE - A - 2 335 107 (GLAXO LABORATORIES) * Claim 1 * --	1	
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TECHNICAL FIELDS SEARCHED (Int. Cl 4)

C 07 C 113/00
C 07 C 109/00
C 07 H
C 07 F

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	03-03-1987	REIF

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